Preparing Kinnex™ libraries using the Kinnex full-length RNA kit

Procedure & checklist

Before you begin

This procedure describes the workflow for constructing Kinnex full-length RNA libraries from total RNA samples for sequencing on PacBio® Sequel® II, Sequel IIe, and Revio™ systems.

Overview	
Samples	1–24
Workflow time	8 hours (for up to 24 samples)
Number of SMRT® Cells per Kinnex library Prep	Up to 2 SMRT Cells for Revio system Up to 4 SMRT Cells for Sequel II/IIe systems

RNA input	
Quality/size distribution	RIN (RNA integrity number) ≥7.0
Quantity	300 ng per library (minimum concentration 43 ng/µL per library)

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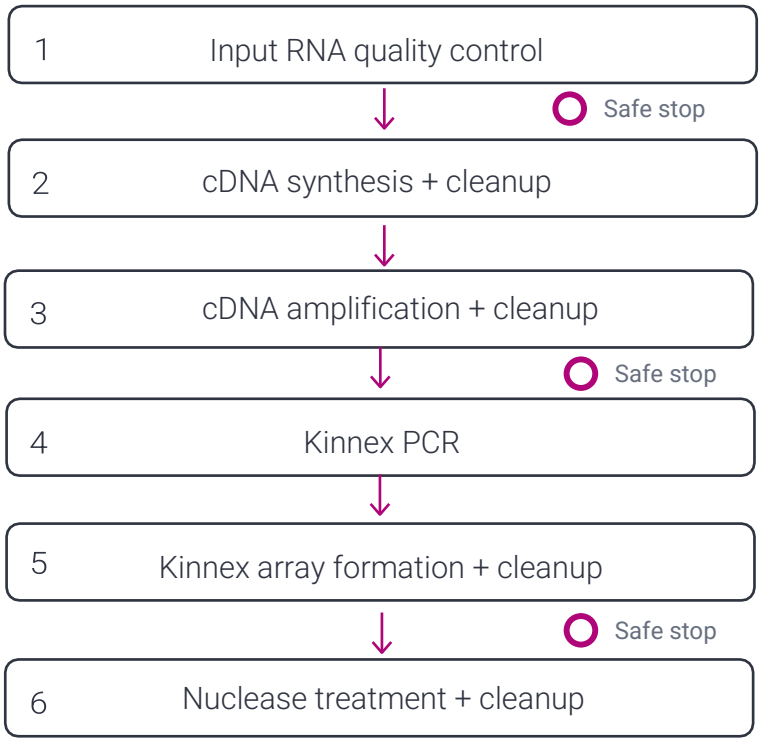
Workflow



Required materials and equipment

RNA and DNA sizing	
2100 Bioanalyzer instrument	Agilent Technologies G2939BA
RNA 6000 Nano kit	Agilent Technologies 5067-1511
Femto Pulse system	Agilent Technologies M5330AA
Genomic DNA 165 kb kit	Agilent Technologies FP-1002-0275
DNA quantitation	
Qubit Fluorometer	Thermo Fisher Scientific Q33238
Qubit 1X dsDNA HS Assay kit	Thermo Fisher Scientific Q33230
Qubit RNA HS Assay kit	Thermo Fisher Scientific Q32852
cDNA synthesis and amplification	
Iso-Seq® Express 2.0 Kit	PacBio® 103-071-500
SMRTbell® cleanup beads	PacBio® 102-158-300*
Elution buffer (50 mL)	PacBio® 101-633-500*
Kinnex Library Prep	
Kinnex PCR 8-fold kit	PacBio® 103-071-600*
Kinnex concatenation kit	PacBio® 103-071-800*

*Part of the Kinnex full-length RNA kit bundle (103-072-000)

Other Supplies	
200 Proof ethanol, molecular biology or ACS grade	Any major lab supplier (MLS)
Nuclease-free water, molecular biology grade	Any MLS (e.g., Sigma-Aldrich W4502)
8-channel pipettes – P20 & P200	Any MLS
Single-channel pipette – P2, P10, P20, P100 or P200	Any MLS
0.2 mL 8-tube strips	USA Scientific TempAssure 1402-4708
Microcentrifuge	Any MLS
Magnetic separation rack compatible with 0.2 mL 8-tube strips	Any MLS (e.g., V&P Scientific, Inc. VP772F4-1)
Thermal cycler compatible with 0.2 mL 8-tube strips	Any MLS
DNA LoBind tubes	Eppendorf 022431021

General best practices

Take care to accurately pipette SMRTbell® cleanup beads because small changes in volume can significantly alter the size distribution of your sample.

Equilibrate the SMRTbell cleanup beads at room temperature for 30 mins prior to use.

In cDNA amplification and Kinnex PCR, keep sample(s) on ice until thermal cycler lid has reached 105°C to avoid digestion of primers by polymerase exonuclease activity.

This workflow takes ~8 hrs to complete. If a stop is necessary, refer to the workflow for safe stopping points.

Multiplexing best practices

Multiplexing can be achieved with one of the three following methods:

1. Barcoded cDNA primers using Iso-Seq primers bc01–12 in step 3 of the protocol. To multiplex, use the Iso-Seq cDNA amplification primer in combination with Iso-Seq primers bc01–12 to amplify samples. After SMRTbell cleanup, Iso-Seq samples can be pooled and brought through a single Kinnex PCR reaction. Each barcoded primer is sufficient for 2 reactions, with the Iso-Seq kit supporting a total of 24 reactions.
2. Barcoded adapters using Kinnex adapters bc01–04. In this case, use barcoded adapters at step 5 "Kinnex array formation" in the workflow.
3. A combination of the above 2 approaches to achieve 48-plex.

Note: if not performing multiplexing, the same Iso-Seq primer barcodes and Kinnex adapter barcodes are still used, but without pooling.

Reagents list

Iso-Seq express 2.0 kit 103-071-500		
	Tube color	Reagent
	Purple	Iso-Seq RT buffer 103-103-900
Kinnex array formation	Orange	Iso-Seq RT primer mix 103-104-000
	Yellow	Iso-Seq RT enzyme mix 103-104-100
	Red	Iso-Seq cDNA PCR mix 103-104-200
	Blue	Iso-Seq template switch oligo 103-104-300
	Green	Iso-Seq cDNA amplification primer 103-104-400
	White	Iso-Seq primer bc01 103-104-500 Iso-Seq primer bc02 103-104-600 Iso-Seq primer bc03 103-104-700 Iso-Seq primer bc04 103-104-800 Iso-Seq primer bc05 103-104-900 Iso-Seq primer bc06 103-105-000 Iso-Seq primer bc07 103-105-100 Iso-Seq primer bc08 103-105-200 Iso-Seq primer bc09 103-105-300 Iso-Seq primer bc10 103-105-400 Iso-Seq primer bc11 103-105-500 Iso-Seq primer bc12 103-105-600

Kinnex PCR 8-fold kit 103-071-600		
	Tube color	Reagent
	Green	Kinnex PCR mix 103-107-700
	Orange	Kinnex primer mix A 103-107-800 Kinnex primer mix B 103-107-900 Kinnex primer mix C 103-108-000 Kinnex primer mix D 103-108-100 Kinnex primer mix E 103-108-200 Kinnex primer mix F 103-108-300

		Kinnex primer mix G 103-108-400
		Kinnex primer mix HQ 103-108-500

Kinnex concatenation kit 103-071-800		
	Tube color	Reagent
	Red	Kinnex enzyme 103-110-400
	Yellow	Kinnex ligase 103-110-500
	White	Kinnex array and repair buffer 103-110-300
	Green	DNA repair mix 103-110-000
	Light Purple	Nuclease buffer 103-110-200
	Light Green	Nuclease mix 103-110-100
	Blue	Kinnex adapter bc01 mix 103-109-600
		Kinnex adapter bc02 mix 103-109-700
		Kinnex adapter bc03 mix 103-109-800
		Kinnex adapter bc04 mix 103-109-900

Workflow steps

1. Input RNA quality control

This protocol requires high-quality RNA. Prior to library preparation, evaluate the size distribution of the input RNA to determine whether it is suitable for the protocol.

✔	Step	Instructions
	1.1	Measure the RNA Integrity Number (RIN) with an Agilent 2100 Bioanalyzer Instrument using the RNA 6000 Nano kit.
	1.2	Proceed to the next step of the protocol if sample quality is acceptable:

RIN	Quality recommendations
≥7.0	Recommended. Proceed to next step of the protocol.
<7.0	Increased library failure rates or reduced data quality.

SAFE STOPPING POINT – Store at -70°C or below

2. cDNA synthesis

2.1 cDNA synthesis

In this step, total RNA samples are converted to first-strand cDNA products.

✔	Step	Instructions
	2.1.1	Quick-spin the Iso-Seq RT enzyme mix in the microcentrifuge to collect liquid, then place on ice.
	2.1.2	Thaw the following components at room temperature, briefly vortex to mix, then quick-spin to collect liquid and place on ice.

Reagent
Iso-Seq RT primer mix (103-104-000)
Iso-Seq RT buffer (103-103-900)
Iso-Seq cDNA PCR mix (103-104-200)
Iso-Seq cDNA amplification primer (103-104-400)
Nuclease-free water
Iso-Seq template switch oligo (103-104-300)
Iso-Seq primer barcodes 01 – 12* (the number of primers thawed will depend on the number of samples processed) 103-104-500 through 103-105-600

*If processing only one sample, any of the 12 Iso-Seq barcoded primers can be used.

2.2 Primer annealing for first-strand synthesis

✔	Step	Instructions
	2.2.1	For each RNA sample to be processed, prepare reagent mix 1 on ice by adding the following components to each tube in the PCR strip tube.

✔	Components	Volume
	Total RNA (300 ng)	<7 µL
	Iso-Seq RT primer mix	2 µL
	Nuclease-free water	Up to 9 µL
	Total volume	9 µL

	2.2.2	Thoroughly mix by pipetting up and down 10 times.
	2.2.3	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	2.2.4	Incubate in a thermal cycler with the following program. Set the lid temperature to 80°C.

Temperature	Time
70°C	5 min
20°C	hold
Proceed immediately to the next step	

2.3 Reverse transcription and template switching

✔	Step	Instructions
	2.3.1	For each RNA sample, prepare reagent mix 2 on ice by adding the following components in the order and volume listed below. Adjust component volumes for the number of samples being prepared, plus 10% overage.

✔	Components	Volume
	Iso-Seq RT buffer (vortex briefly before use)	5 µL
	Nuclease-free Water	3 µL
	Iso-Seq RT enzyme mix	2 µL
	Total volume added per reaction	10 µL

	2.3.2	Pipette-mix and quick-spin in a microcentrifuge to collect all liquid.
	2.3.3	Add 10 µL of reaction mix 2 to the 9 µL from reaction mix 1 (Section 2.2) for a total volume of 19 µL.

✔	Tube	Reagent	Volume
	Previous	Reagent mix 1 from step 2.2	9 µL
		Reagent mix 2	10 µL
	Total volume added per reaction		19 µL

2.3.4 Thoroughly mix by pipetting up and down 10 times and then quick-spin to collect all liquid.

2.3.5 Incubate in a thermocycler with the following program. Set the lid temperature to 52°C.

Temperature	Time
42°C	45 min
20°C	Hold

Proceed immediately to the next step.

2.3.6 Remove the sample tube from the thermal cycler and add 2 µL of Iso-Seq template switch oligo to the 19 µL reaction at room temperature for a total volume of 21 µL. Mix by pipetting up and down 10 times and then quick-spin to collect all liquid from the sides of the tube.

2.3.7 Return sample tube to thermal cycler and incubate with the following program. Set the lid temperature to 52°C.

Temperature	Time
42°C	15 min
4°C	hold

2.4 1.3X SMRTbell bead cleanup

✔	Step	Instructions
	2.4.1	For each sample, add 29 µL of elution buffer to the 21 µL reverse transcription and template switching reaction (Section 2.3) for a total volume of 50 µL.
	2.4.2	Add 65 µL of resuspended, room-temperature SMRTbell cleanup beads.
	2.4.3	Mix beads by pipetting 10 times or until evenly distributed.
	2.4.4.	Quick-spin strip tubes in a microcentrifuge to collect liquid.
	2.4.5	Leave at room temperature for 10 minutes to allow DNA to bind the beads.
	2.4.6	Place the strip tubes in a magnetic separation rack until the beads separate fully from the solution.
	2.4.7	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	2.4.8	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into the strip tube. After 30 seconds, pipette off the 80% ethanol and discard.
	2.4.9	Repeat the previous step.
	2.4.10	Remove residual 80% ethanol: • Remove the strip tube from the magnetic separation rack. • Quick-spin the strip tube in a microcentrifuge.

- Place the strip tube back in a magnetic separation rack until beads separate fully from the solution.
- Pipette off residual 80% ethanol and discard.

2.4.11 Remove the strip tube from the magnetic rack. Immediately add 21 µL of elution buffer to the strip tube and resuspend the beads by pipetting 10 times or until evenly distributed.

2.4.12 Quick-spin the strip tube in a microcentrifuge to collect liquid.

2.4.13 Leave at room temperature for 5 minutes to elute the DNA.

2.4.14 Place the strip tube in a magnetic separation rack until the beads separate fully from the solution.

2.4.15 Slowly pipette off the cleared supernatant without disturbing the beads. Transfer 21 µL of the supernatant to a new strip tube. Discard the old strip tube with beads.

2.4.16 Proceed to the next step of the protocol.

3. cDNA amplification

First-strand cDNA products are PCR-amplified and barcoded using barcoded Iso-Seq primers at this step.

3.1 cDNA amplification

✔	Step	Instructions
	3.1.1	For each sample, prepare reaction mix 3 on ice by adding the following components in the order and volume listed below. Adjust component volumes for the number of samples being prepared, plus 10% overage. Pipette mix master mix. Iso-Seq primer bc01–12 will be added to each sample individually and should not be added to the master mix.

✔	Components	Volume
	Iso-Seq cDNA PCR mix	25 µL
	Iso-Seq cDNA amplification primer	2 µL
	Total volume	27 µL

	3.1.2	On ice, add 27 µL of reaction mix 3 to the 21 µL of the eluted cDNA (from previous Section 2.4). Add 2 µL of Iso-Seq primer bc01–12 for a total volume of 50 µL.
	3.1.3	Thoroughly mix by pipetting up and down 10 times and then quick spin to collect all liquid.
	3.1.4.	Run the thermal cycler program below with the lid temperature set to 105°C. Keep sample on ice until thermal cycler lid has heated to 105°C.

PCR program	
45 seconds at 98°C	1 cycle
10 seconds at 98°C 15 seconds at 60°C 3 minutes at 72°C	10 cycles
5 minutes at 72°C	
Hold at 4°C	

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

3.2 Cleanup of amplified cDNA using 0.9X SMRTbell Cleanup beads

✔	Step	Instructions
	3.2.1	Add 45 µL (0.9x) of resuspended, room-temperature SMRTbell cleanup beads to the 50 µL of cDNA amplified reaction from Section 3.1. **The correct ratio of beads to sample is critical at this step.**
	3.2.2	Mix beads by pipetting 10 times or until evenly distributed.
	3.2.3	Quick-spin strip tubes in a microcentrifuge to collect liquid.
	3.2.4	Leave at room temperature for 10 minutes to allow DNA to bind beads.
	3.2.5	Place the strip tubes in a magnetic separation rack until beads separate fully from the solution.
	3.2.6	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	3.2.7	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into the strip tube. After 30 seconds, pipette off the 80% ethanol and discard.
	3.2.8	Repeat the previous step.
	3.2.9	Remove residual 80% ethanol: • Remove the strip tube from the magnetic separation rack. • Quick-spin the strip tube in a microcentrifuge. • Place the strip tube back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	3.2.10	Remove the strip tube from the magnetic rack. Immediately add 24 µL of elution buffer to the strip tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	3.2.11	Quick-spin the strip tube in a microcentrifuge to collect liquid.
	3.2.12	Leave at room temperature for 5 minutes to elute DNA.
	3.2.13	Place the strip tube in a magnetic separation rack until the beads separate fully from the solution.
	3.2.14	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer 24 µL of the supernatant to a new strip tube. Discard the old strip tube with beads.
	3.2.15	Recommended: Measure concentration and size distribution of each cDNA sample. • Take a 1 µL aliquot from each strip tube. Dilute each aliquot with 4 µL of elution buffer. • Measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. • Dilute 1:4 dilution further to 1.5 ng/µL based on the Qubit reading if needed. • Run 1 µL on an Agilent Bioanalyzer using a High Sensitivity DNA kit.
	3.2.16	The expected recovery after cDNA amplification SMRTbell clean-up is >100 ng. A minimum of 55 ng of total cDNA is recommended to proceed with Kinnex PCR (Step 4). If less than 55 ng but

		more than 25 ng is recovered, proceed with Kinnex PCR but expect lower yields. Do not proceed with less than 25 ng.

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

3.3 Pooling barcoded cDNA (skip if not multiplexing)

✔	Step	Instructions
	3.3.1	Using the concentration reading from the Qubit fluorometer, pool an equal mass of each barcoded cDNA sample for a total mass of 55 ng. Store any remaining purified, amplified barcoded cDNA at 4°C for future use.
	3.3.2	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	3.3.3	Proceed to next step of the protocol.

4. Kinnex PCR & pooling

4.1 Kinnex PCR

Perform 8 parallel Kinnex PCR reactions with Kinnex primers to generate DNA fragments containing orientation-specific Kinnex segmentation sequences.

✔	Step	Instructions
	4.1.1	Thaw primers. The entire volume of primers can be transferred to an 8-tube strip for ease of use with a multi-channel pipette.

	8X concatenation	Tube color	P/N
1	Kinnex primer mix A	Orange	103-107-800
2	Kinnex primer mix B	Orange	103-107-900
3	Kinnex primer mix C	Orange	103-108-000
4	Kinnex primer mix D	Orange	103-108-100
5	Kinnex primer mix E	Orange	103-108-200
6	Kinnex primer mix F	Orange	103-108-300
7	Kinnex primer mix G	Orange	103-108-400
8	Kinnex primer mix HQ	Orange	103-108-500

	4.1.2	Briefly vortex to mix, then quick-spin to collect liquid and place the primer mixes on ice.
	4.1.3	Thaw the following components, briefly vortex to mix, then quick-spin to collect liquid and place on ice. Add the components on ice in a LoBind tube.

Master mix components	Volume for 8X concatenation*
PCR-grade water	88-X µL
Kinnex PCR mix (103-107-700)	110 µL
55 ng of amplified cDNA from Step 3.2.16	X µL
Total volume	198 µL

X = 55 (ng)/purified pooled DNA concentration from step 3.2.16 (single-plex) or step 3.2.3 (multiplex)

*10% overage included

	4.1.4.	Aliquot **22.5 µL** of Master Mix 1 into each of the 8 PCR tubes (for 8X concatenation).
	4.1.5	Add **2.5 µL** of Kinnex primer mix into each of 8 PCR tubes from Step 4.4.
	4.1.6	Set up the thermal cycler program listed below with the lid set to 105˚C. **Keep sample(s) on ice until the lid is heated to 105°C.** The duration of PCR is approximately 1 hour.

Step	Temperature	Duration	Cycles
Initial denaturation	98°C	3 min	1
Denaturation	98°C	20 s	9
Annealing	68°C	30 s	
Extension	72°C	4 min	
Final extension	72°C	5 min	1
Hold	4°C	Hold	

4.2 Pooling of 8 Kinnex PCR products and 1.05X SMRTbell cleanup

✔	Step	Instructions
	4.2.1	Add 23 µL from each of the 8 PCR reactions into a 1.5 mL tube for a total volume of 184 µL. An equal volume of each PCR product is necessary for efficient array assembly.
	4.2.2	Add 193 µL (**1.05X** v/v) of resuspended, room-temperature SMRTbell cleanup beads to a tube of pooled Kinnex PCR amplicon. **The correct ratio of beads to pooled sample is critical at this step.**
	4.2.3	Pipette-mix the beads until evenly distributed.
	4.2.4	Quick-spin the tube in a microcentrifuge to collect liquid.
	4.2.5	Leave at **room temperature** for **10 minutes** to allow the DNA to bind beads
	4.2.6	Place the tube in a magnetic separation rack until the beads separate fully from the solution.
	4.2.7	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	4.2.8	Slowly dispense **200 µL**, or enough to cover the beads, of **freshly prepared 80% ethanol** into the tube. After **30 seconds**, pipette off the 80% ethanol and discard.
	4.2.9	Repeat the previous step.
	4.2.10	Remove residual 80% ethanol: • Remove the tube from the magnetic separation rack. • Quick-spin the tube in a microcentrifuge. • Place the tube back in the magnetic separation rack until the beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	4.2.11	Remove the tube from the magnetic rack. **Immediately** add **40 µL** of **elution buffer** to the tube and resuspend the beads by pipetting 10 times or until evenly distributed.

	4.2.12	Quick-spin the tube in a microcentrifuge to collect liquid.
	4.2.13	Leave at **room temperature** for **5 minutes** to elute DNA.
	4.2.14	Place tube in a magnetic separation rack until beads separate fully from the solution.
	4.2.15	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a **new LoBind tube**. Discard old tube with beads.
	4.2.16	Make a 1:10 dilution of the sample in elution buffer and measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Typical yield is 6–12 µg.

5. Kinnex array formation

5.1 Kinnex array formation

In this step, treat PCR-amplified cDNA fragments from Step 4.2 with Kinnex enzyme, ligase, and barcoded Kinnex terminal adapters to assemble cDNA segments into a linear array.

✔	Step	Instructions
	5.1.1	In a 0.2 mL PCR tube, add 4–8 µg of sample from Step 4.2.15, in 39 µL of volume (102–205 ng/µL). Dilute with elution buffer going into this step if the sample is too concentrated. Add 2 µL of Kinnex adapter bc01–04 (select one barcode per library preparation). Note: if combining multiple barcoded Kinnex libraries for sequencing, make sure each library uses one of the 4 different Kinnex barcoded adapters.
	5.1.2	Add the following components in the listed order. If processing multiple samples, make a master mix with 10% overage. Pipette mix master mix.

Components	Volume
Kinnex array and repair buffer (103-110-300)	7.0 µL
Kinnex enzyme (103-110-400)	4.0 µL
Kinnex ligase (103-110-500)	6.0 µL
Total RM1 volume	17 µL

Add 17 µL of master mix to the PCR tube containing 39 µL of sample (102–205 ng/µL) and 2 µL of Kinnex adapter. Pipette-mix and run the Kinnex primer digestion/ligation program with the lid set to 55°C.

5.1.3

Step	Temperature	Duration
1	45°C	60 min
2	4°C	Hold

	5.1.4	After running the Kinnex primer digestion/ligation program, add 2 µL of DNA repair mix directly to the Kinnex primer digestion/ligation sample.
	5.1.5	Thoroughly mix by pipetting up and down 10 times and then quick spin to collect all liquid.

	5.1.6	Run the DNA Damage Repair Program with the lid set to >55°C.

Step	Temperature	Duration
1	45°C	30 min
2	4°C	Hold

5.2 1X SMRTbell bead cleanup

Cleanup with 1X SMRTbell cleanup beads

✔	Step	Instructions
	5.2.1	Add 1X v/v (60 µL) of resuspended, room temperature SMRTbell cleanup beads to each sample.
	5.2.2	Pipette-mix the beads until evenly distributed and quick-spin in a microcentrifuge to collect liquid.
	5.2.3	Leave at room temperature for 10 minutes to allow the DNA to bind the beads.
	5.2.4	Place the tube strip in a magnetic separation rack until the beads separate fully from the solution.
	5.2.5	Slowly pipette off the cleared supernatant without disturbing the beads. Discard the supernatant.
	5.2.6	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.
	5.2.7	Repeat the previous step.
	5.2.8	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	5.2.9	Remove the tube strip from the magnetic rack. Immediately add 40 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed. Quick-spin the tube strip in a microcentrifuge to collect liquid.
	5.2.10	Leave at room temperature for 5 minutes to elute DNA.
	5.2.11	Place the tube strip in a magnetic separation rack until the beads separate fully from the solution.
	5.2.12	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new PCR strip tube. Discard old tube with beads.

SAFE STOPPING POINT -- Store at 4°C or -20°C for long-term storage

6. Nuclease treatment & cleanup

6.1 Nuclease treatment

✔	Step	Instructions
	6.1.1	Add the following components listed below to a new microcentrifuge tube. Adjust component volumes for the number of samples being prepared, plus 10% overage. Pipette mix master mix. For individual preps, add components directly to each sample from the previous step in the order and volume listed below.

Nuclease master mix

Tube	Component	Volume
Light purple	Nuclease buffer (103-110-200)	5 µL
Light green	Nuclease mix (103-110-100)	5 µL
	Total volume	10 µL

	6.1.2	Add 10 µL of Nuclease Master mix to each sample. The total volume should be 50 µL.
	6.1.3	Thoroughly mix by pipetting up and down 10 times and then quick spin to collect all liquid.
	6.1.4	Run the nuclease treatment program with the lid set to >47°C.

Step	Temperature	Duration
1	37°C	15 min
2	4°C	Hold

6.2 Final cleanup with SMRTbell cleanup beads

✔	Step	Instructions
	6.2.1	Add 50 µL SMRTbell cleanup beads to each sample from the previous step. Pipette-mix the beads until evenly distributed.
	6.2.2	Quick-spin the tube strip in a microcentrifuge to collect all liquid.
	6.2.3	Leave at room temperature for 10 minutes to allow DNA to bind the beads.
	6.2.4	Place the tube strip in a magnetic separation rack until the beads separate fully from the solution.
	6.2.5	Slowly pipette off the cleared supernatant without disturbing the beads and discard the supernatant.
	6.2.6	Slowly dispense 200 µL, or enough to cover the beads, of freshly prepared 80% ethanol into each tube. After 30 seconds, pipette off the 80% ethanol and discard.
	6.2.7	Repeat the previous step.
	6.2.8	Remove residual 80% ethanol: • Remove the tube strip from the magnetic separation rack. • Quick-spin the tube strip in a microcentrifuge. • Place the tube strip back in a magnetic separation rack until beads separate fully from the solution. • Pipette off residual 80% ethanol and discard.
	6.2.9	Remove the tube strip from the magnetic rack. Immediately add 20 µL of elution buffer to each tube and resuspend the beads by pipetting 10 times or until evenly distributed.
	6.2.10	Quick-spin the tube strip in a microcentrifuge to collect liquid.
	6.2.11	Leave at room temperature for 5 minutes to elute DNA.
	6.2.12	Place the tube strip in a magnetic separation rack until the beads separate fully from the solution.
	6.2.13	Slowly pipette off the cleared supernatant without disturbing the beads. Transfer supernatant to a new 0.5 mL LoBind tube or PCR tube strip. Discard old tube strip with beads.
	6.2.14	Take a 1 µL aliquot from each tube. Make a 1:5 dilution of the sample in elution buffer and measure DNA concentration with a Qubit fluorometer using the 1x dsDNA HS kit. Calculate the total mass. Expect 10-25% recovery of the starting Kinnex-PCR product. Recommended: Further dilute each aliquot to 250 pg/µL with the Femto Pulse dilution buffer. Measure the final SMRTbell library size distribution with a Femto Pulse system.
	6.2.15	Proceed to SMRT Link Sample Setup to prepare the SMRTbell library for sequencing.
	6.2.16	Store SMRTbell libraries at 4°C if sequencing within the week. Long-term storage should be at -20°C. Minimize freeze-thaw cycles when handling SMRTbell libraries.

PROTOCOL COMPLETE

Revision history (description)	Version	Date
Initial release.	01	October 2023